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ORIGINAL ARTICLE

Mycelium of fungi isolated from mouldy foods inhibits *Staphylococcus aureus* including MRSA – A rationale for the re-introduction of mycotherapy?

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Abstract Fungal mycelium capable of producing antibacterial agents was isolated from samples of apple, beetroot, lemon and orange; the mycelium of all isolates produced penicillin, while the apple and beetroot samples also produced the antibacterial mycotoxin patulin. The known penicillin-producing fungi were shown to produce penicillin, but not patulin. The mycelial discs of all of fruit and vegetable isolates, as well as the two known penicillin producing fungi, inhibited *Staphylococcus aureus*, and mycelium of all isolates inhibited MRSA, in contrast, only one of the two known penicillin-producers did so. The results are discussed in relation to the possibility of using the mycelium of *Penicillium* species in mycotherapy.

1. Introduction

Moulds (i.e. filamentous fungi) were widely used as curative agents in all of the world's cultures well before Fleming's famous discovery of penicillin in 1928. Imhotep, an ancient Egyptian practitioner, for example, used mouldy bread to treat infections of the face (Wainwright et al., 1992). The literature from more recent folk medicine has documented some other examples of the use of moulds on infections. For example, mouldy jam and mouldy bread were widely used in folk-based therapy in Quebec (Canada), Devon (UK), and Kansas (USA) and poultices made from mouldy chewed barley and apple have long been used in Asia to cure surface wounds.

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In 1640, one of London apothecaries also advised that moulds have a curative effect when applied to infections (Wainwright, 1989).

The difficulties facing researchers attempting the large scale production of penicillin during 1940s encouraged some hospital scientists to re-evaluate the therapeutic properties of crude penicillin filtrates (Wainwright et al., 1992; Dunayer et al., 1994; Enoch and Wallersteiner, 1994; Robinson and Wallace, 1943; Wainwright, 1987, 1994, 1998); a number of different methods were used including the topical application of crude penicillin involving (a) the application of bandages saturated with liquid filtrates or (b) dressings inoculated with the penicillin-producing mould *Penicillium notatum* (Wainwright, 1987). Although many successful cures were achieved using crude penicillin, its unregulated production and potential problems relating to contamination and purity caused concern and, not surprisingly, it became completely redundant once purified penicillin became widely available (Wainwright, 1987). Wainwright et al. (1992) investigated the scientific basis of the mycotherapy and their results suggest that the active agent responsible for the observed curative effects is patualin, and not penicillin.

The aim of the work reported here was to determine if fungal mycelium, grown from moulds isolated from mouldy foods, is (1) able to produce both penicillin and patulin and (2) whether they produce agents capable of inhibiting the growth of *Staphylococcus aureus* and MRSA. It is hoped that these studies will give impetus to medical scientists to consider the re-introduction of mycotherapy into modern medicine.

2. Experimental section

The following test bacteria were used: methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA); unless stated otherwise, the organisms were obtained from the Departmental Culture Collection. A variety of naturally mouldy vegetables and fruits were collected from local retail outlets; i.e. apple, oranges, lemons and beetroot. Two known penicillin-producing fungi, *Penicillium chrysogenum* (IMI 37767) and *P. chrysogenum* (IMI 24317), were also included.

The fungi were isolated from a variety of collected mouldy foods. Either skin (2 × 2 mm) or heavily sporulating colonies were picked off, to Czapek Dox liquid medium (Oxoid) and incubated at 25 °C with shaking 150 rpm for two weeks.

The isolated fungal cultures were then identified after visualisation under a low power microscope and grown as described in the above method. The mycelia of these cultures were separated by filtering using Whatman filter paper. The antibacterial activity of the harvested mycelia was then determined by placing a disc of fungal mycelia on Nutrient Agar (Oxoid) plates which had been previously covered with a culture of either MSSA or MRSA. Plates were incubated at 37 °C for 24 h. The diameter of the inhibition zone (mm) produced was then measured.

The contents of all growth fungi liquid culture flasks were filtered using Whatman No. 1 filter paper and the mycelium was transferred into 250 ml separating funnel contained two equal volumes of ethyl acetate. The separating funnel was shaken for 2 min and the contents were allowed to settle and layers separated. The bottom aqueous (fungal culture) layer was removed and discarded, while the top layer (ethyl acetate) was collected and combined with 2 g of anhydrous sodium sulphate (Na_2SO_4), then left to evaporate to dryness.

Patulin was detected using thin layer chromatography (Gimeno, 1979; Gimeno and Martings, 1983), Sigma–Aldrich® TLC plates, cellulose matrix, H × W 10 cm × 20 cm with a fluorescent indicator were used as a stationary phase. Two 10 μl aliquots of extracts were spotted along a line 3 cm from the lower edge of the plate. 5 μl of patulin standard were spotted as a reference standard solution. The plates were then placed in Latch-lid™ TLC chambers (Aldrich) for one dimensional development for about 2 h in benzene:methanol:acetic acid – BMA (90:5:5 v/v/v)(mobile phase); they were then left at room temperature until the solvent front had reached a line marked 2 cm from the top of the plate. The plates were then removed and air-dried in a fume cabinet and sprayed with a freshly prepared mixture of 0.5 ml *p*-anisaldehyde (in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulphuric acid) and then heated at 110° in a hot-air oven for 10 min. Plates were then were visualised using a 2UV Transilluminator (300–360 nm).

After being air-dried and sprayed with a fresh preparation of *p*-anisaldehyde (Fluka), developed chromatograms were viewed under visible and UV light for the presence of fluorescent spots. The *p*-anisaldehyde spray allows patulin to be detected as faint brown spots under visible light and as yellow-orange under UV light. According to Scott et al., 1970 using ethanol instead of methanol in the spray reagent will improve the detection of patulin at a detection limit of 0.1 μg (Scott and Somers, 1968; Scott et al., 1970).

The SNAP MRL Beta-Lactam Kit (IDEXX, USA) was used to test for the presence of betalactams; it depends on an enzyme-linked receptor-binding assay, designed to detect most beta-lactam antibiotics, notably penicillin G. The Kit contains: a SNAP Device, a sample tube containing reagent pellet, a pipette, SNAP positive controls and a heater block capable of maintaining an operating temperature of 45 °C + 5 °C.

The heater block used was preheated to 45 °C + 5 °C, the SNAP device, the pipette and sample tube were removed from the bag and the reagent pellet was checked to be present at the bottom of the sample tube. The SNAP device was then placed in the heater block; 2 ml of the liquid culture (sample), shaken thoroughly, and then the sample tube cap was removed and discarded, 450 μl of the sample was drawn up with the IDEXX pipette, the sample was then added carefully from the pipette to the tube. To dissolve the reagent pellet the sample tube was shaken. The sample tube was then incubated in the preheated heater block at 45 °C for 5 min.

The contents of the sample tube were poured into the sample well of the SNAP device, and the tube was discarded. The sample flowed across the results window towards the blue activation circle. When the blue activation circle began to disappear, the activator button was pushed firmly until it snapped flush with the body of the SNAP device, during colour development the SNAP device was left in the heater block; after 4 min, the result was ready to be read.

3. Results and discussion

The ability of mycelium of *Penicillium* species to inhibit both methicillin sensitive (MSSA) and methicillin resistant *S. aureus*

(MRSA) is shown in Table 1; in the case of the apple isolate, an illustration of the zones produced on the test plate is also shown in Fig. 1. As expected, a paper test disc containing penicillin was shown to inhibit MSSA, but not MRSA. Mycelium from all of the *Penicillium* cultures isolated from foods inhibited both MSSA and MRSA. The obvious question is what antibacterial agent is responsible for this inhibition? Tests using SNAP (Fig. 2) to test for penicillin and TLC for patulin showed that penicillin was found in culture filtrates from all of the individual fungi, while patulin was produced by the apple and beetroot isolates. The isolation of a patulin-producing fungus from apples is to be expected since patulin is commonly produced on both apples and in apple juice (Brian et al., 1956; Bilai, 1963; Wilson and Nuovo, 1973).

Although penicillin and patulin were tested here it is, of course, possible that other antibacterial agents were also produced which act alone, or together with penicillin or patulin to inhibit the MSSA or MRSA. This would explain how the MRSA was inhibited when this organism is known to be resistant to penicillin. In relation to mycotherapy, one could ask the question-is the nature of the antibacterial agent or agents produced by the mycelium important? Certainly from a normal clinical viewpoint, a clinician would be expected to know what agents are being produced by a mould which is being used to treat a wound. This is especially true in the case of *Penicillium* species which are known to produce various potentially dangerous toxic mycotoxins. Patulin, for example, is

Table 1 Antibacterial activity of the mycelial discs (zone of inhibition, mm) of *Penicillium* cultures and isolates from foodstuffs.

Test	MSSA	MRSA	Penicillin	Patulin
Penicillin G disc (6 μg)	30	0	+	–
P. chrysogenum (IMI 24317)	26	0	+	–
P. chrysogenum (IMI 37767)	14	15	+	–
Penicillium sp. (apple)	16	17	+	+
Penicillium sp. (beetroot)	17	18	+	+
Penicillium sp. (lemon)	18	17	+	–
Penicillium sp. (orange)	19	18	+	–

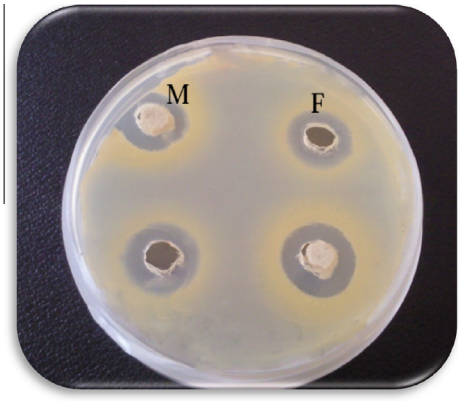

Figure 1 The antibacterial activity of mycelia (M) and the filtrates (F) of the fungus was isolated from apple liquid culture against MRSA.

Figure 2 The SNAP device showing different results according to samples sources. Note in positive control, lemon and beetroot samples, the control spots are darker than the sample spot (positive results), whereas in the negative control (sterilised Czapek Dox liquid medium) the control spot was lighter than the sample spot.

toxic and its long term intake into the body may lead to the formation of cancers (Reddy et al., 1978). As was discussed in the Introduction, mycelium of Fleming's penicillin-producing mould was successfully applied to infected wounds during the mid to late 1940s, where it proved to be highly successful, although its use soon relegated to a historical artefact once purified penicillin became widely available. There appears therefore following controlled clinical trials, such mycotherapy might be re-introduced into modern medicine especially since some, newly isolated, penicillin-producing moulds might produce antibacterial agents other than penicillin, which might inhibit the growth of MRSA in infected wounds. During clinical experience gained in the period when *P. notatum* was used as a filtrate, as macerated mycelium in filtrates, or when growing on agar or other substrates applied to wounds exposed no examples of toxic contradictions (Wainwright et al., 1992). The application to wounds, of a mould which is known to produce penicillin and or patulin, as well as other unknown antibacterial (or otherwise) compounds would obviously be initially dismissed by regulatory authorities and would not pass ethical panel scrutiny.

Under what conditions might moulds, isolated from common foods, be used to treat wounds infected with *S. aureus*, including MRSA?

Clearly then, a pure culture of a *Penicillium*, known to produce penicillin and not patulin or other toxicants (like the two IMI strains used here) could be safely used to treat wound infections in hospitals at the present time. Finally, can a

Figure 3 Simulated treatment of a wrist wound with surface grown mycelium of an antibacterial agent-producing strain of a *Penicillium* species.

situation be imagined in which the results obtained in this study could be put to practical use?

The obvious answer is that *Penicillium* moulds isolated from mouldy foods could be applied to wounds *in extremis*, that is when no other medical intervention is available to treat an infected wound, e.g. following an event which leads to the total breakdown of society (e.g. after a nuclear war, or during local extreme living conditions such as in prisoner of war camps. Under these conditions, *Penicillium* cultures could be isolated from mouldy foods (a mixture of such moulds from various foods might prove particularly effective) and could be applied to wounds (Fig. 3) in the knowledge that they would contain penicillin and or patulin, plus other antibacterial metabolites. Under such extreme condition such metabolites could prove the difference between life and death for a patient suffering from a critically infected wound. Alternatively, under these circumstances food-related moulds could be applied prophylactically to prevent the development of an infection in an open wound.

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